Spatial and temporal analysis of microbial populations in production broiler house litter in the southeastern United States[1]

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Primary Audience: Broiler Production Managers, Poultry Researchers, Environmental Microbiologists

SUMMARY

The main objectives of this study were to discern intrahouse spatial and temporal effects on foodborne and nuisance pathogen bacterial levels in actively used commercial broiler litter. The purpose of the study was to provide critical information regarding microbial hot spots, which may be targeted for site-specific litter treatments. A single broiler-concentrated animal feeding operation was monitored throughout 3 consecutive flocks. *Salmonella enterica*, *Listeria monocytogenes*, *Staphylococcus* spp., *Enterococcus* spp., *Clostridium perfringens*, and *Campylobacter* spp. were monitored at specific locations. Additionally, antibiotic resistance characteristics were quantified from bacterial isolates. *Clostridium perfringens*, *Staphylococcus* spp., and *Enterococcus* spp. were consistently present at levels of 7 $\log_{10}$, 12 $\log_{10}$, and 8 $\log_{10}$ cfu/kg of litter, respectively; whereas *S. enterica*, *Campylobacter* spp., and *L. monocytogenes* were not present or present at low levels compared with other bacteria investigated. Temporally, *S. enterica* was found early in the flock, whereas *C. perfringens*, *Staphylococcus* spp., and *Enterococcus* spp. levels were greater later in the flock. The effect of flock cycle was noted for *S. enterica* and *L. monocytogenes,* which were found at greater frequency with the first flock (summer). *Salmonella enterica* was more commonly associated with the end walls, but overall it appeared that pathogen levels were difficult to predict.

Key words: *Salmonella*, *Campylobacter*, *Staphylococcus*, *Enterococcus*, *Listeria*, poultry, litter, broiler

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DESCRIPTION OF PROBLEM

Approximately 8 billion broilers are produced per year in the United States [1], with about 10% of those produced in Mississippi [2]. The demands of the growing market are met by large broiler farms, classified as concentrated animal feeding operations, capable of producing over 100,000 broilers per house per year (5–6 flocks). Investigating site-dependent locations of pathogenic and nuisance microbial communities within the broiler house environment may lead to increased broiler productivity by establishing target areas within the house aimed at pathogen treatment. The pathogen and nuisance microbial populations in these environments can be detrimental to food animal industries [3], especially given the concerns for food safety and animal health concerns. *Salmonella* spp., *Campylobacter* spp., and *Listeria* spp. are foodborne pathogens that may be found in the broiler house environment [3]. These 3 zoonotic pathogens are responsible for the majority of bacterial foodborne diseases and fatalities in the United States [4]. *Salmonella* is a major concern in the poultry industry and is responsible for several poultry-associated human disease outbreaks [5–7]. The microbial ecology of the litter can affect broiler health during production and may affect public health by facilitating pathogen carriage and transmission during production and processing [8]. Marin et al. [9] investigated common risk factors capable of introducing *Salmonella* into the house; these risks included chick delivery box liners, farmers' boots, and broiler feed. Volkova et al. [8] determined that the presence of *Salmonella* in litter before flock placement and throughout grow-out contributed to its presence on post-chill tank carcasses during processing. Reducing pathogen levels by targeting specific problem areas within the house may be a means to reduce broiler mortality and curtail the spread of zoonotic pathogens.

Increased scrutiny and criticism of antibiotic use in animal agriculture requires alternative strategies to reduce bacterial pathogens. Alternative methods to reduce pathogens in broiler litter have been investigated [10], but few have demonstrated effective long-term reduction. These alternative methods may, however, be more effective if data were available to guide decisions on site-specific treatments. The primary objective of this study was to determine bacterial profiles in broiler house litter, with an emphasis on *Salmonella enterica* and other pathogens, as affected by environment, management, and spatial and temporal variables. The goal of the research was to provide site- and pathogen-specific data that would allow for better informed decisions and improve future control of microbial populations in broiler house litter.

MATERIALS AND METHODS

House Litter Management

A previously instrumented commercial broiler house (located in north central Mississippi) was selected for this study [11, 12]. The broiler farm comprised 8 broiler houses approximately 12.8 × 152.4 m, housing approximately 26,000 to 28,000 broilers per house per flock. The flock cycle comprised 6.5 wk from placement to removal, with 2 wk between flocks. The top 10 cm of litter was removed after each flock and the remaining litter was dressed with fresh pine shavings in preparation for the next flock.

Litter Sample Collection

Litter samples (100 g) were collected during the flock cycle throughout the house using a 2 dimensional grid corresponding to the water lines (**WL**), feeder lines (**FL**), western side walls (**SW**), and northern and southern end walls (**EW**) of the house. The specific determination of litter collection was associated with equidistant grid points outlined near these locations on both halves of the house, brood and nonbrood. Litter samples were collected from June to December of 2008 comprising 3 consecutive flocks in the same corresponding locations. Flock 1 was sampled from June 16 to July 28, flock 2 from August 25 to October 6, and flock 3 from October 27 to December 10 and are referred to as summer, fall, and winter flocks, respectively. Sixteen litter samples were collected biweekly (0, 2, 4, and 6 wk) for each represented flock. Two replicate samples were collected from each of 8 locations, 4 sites on each half (north, brood end and south, nonbrood end) of the house. Litter was collected in Whirl-Pak bags [13] and

transported in a cooler that contained icepacks to keep samples cool until processing at the laboratory. Litter temperature was monitored and recorded continuously throughout the study at WL, FL, EW and SW locations in the south half of the house with a HOBO H21–002 microstation logger [14]. Litter moisture content was determined for each litter sample by weighing 10 g initially, drying at 104°C for 48 h, and reweighing to measure dry weight. Litter moisture content was determined by the difference.

Litter Processing

All samples were processed within 24 h of collection. To ensure sample homogeneity, litter was blended with an industrial stainless steel blender [15] for a minimum of 30 s. Between each sample, the blender was cleaned with 70% ethanol and rinsed with sterile deionized water. Prior to microbial analyses, 10 g (wet weight) of poultry litter was suspended in 95 mL of sterile physiological saline, stomached for 30 s, and serially diluted for analysis as described by Brooks et al. [11]. The litter-saline mixture taken from the stomach bags was considered the 1/10 dilution.

Microbial Assays

Staphylococcus spp. (standard plating), *Enterococcus* spp. and *C. perfringens* (membrane filtrations), *Campylobacter* spp. and *L. monocytogenes* (presence/absence), and *Salmonella* spp. [most probable number (**MPN**)] were all assayed. *Staphylococcus* spp. were assayed in duplicate by spread-plating 0.1 mL of a proper dilution on manitol salt agar [16] and incubated at 35°C for 24 to 48 h. *Enterococcus* spp. were analyzed on mEnterococcus agar [17], incubated at 35°C for 24 h, transferred to bile-esculin agar [18], and incubated for an additional hour at 35°C. *Clostridium perfringens* samples were heated to 70°C for 10 min before membrane filtration and placed on mCP agar [19]. Samples were incubated at 44.5°C for 24 h under anaerobic conditions created by an Anoxomat gas generation system [20]. All mCP plates were exposed to ammonium hydroxide fumes for a minimum of 30 s for confirmation of presumed positive *C. perfringens* colonies. Only colonies that turned pink once exposed were considered *C. perfringens*. Randomly selected colonies were further confirmed by streaking each to 5% sheep (*Ovisaries*) blood [21] tryptic soy agar [22], anaerobically incubating at 44.5°C, and noting the characteristic double zone of hemolysis.

For cultural analysis of *Campylobacter* spp. and *L. monocytogenes* in broiler litter, pre-enrichments were performed by adding 10 g of broiler litter, respectively, to 95 mL of *Campylobacter* enrichment broth [23], and to 95 mL of University of Vermont (modified)–*Listeria* enrichment broth [24]. *Campylobacter* enrichment broth was incubated microaerophillically at 35°C for 4 h then moved to 42°C for 44 h. *Campylobacter* was streaked for isolation onto 5% sheep blood tryptic soy agar and incubated microaerophillically at 42°C for 48 h. A microaerophillic environment was achieved using the Anoxomat gas system as described above by placing inoculated media in chambers that reduce oxygen levels to a gas mixture of H:N:CO_2 at a ratio of 10:80:10 [25]. For *L. monocytogenes* isolation, UVM was incubated at 30°C for 48 h. For each sample, triplicate 0.1-mL aliquots were transferred to 10 mL of Fraser's broth tubes [26] and incubated at 35°C for 24 h. Positive tubes were streaked onto modified Oxford agar [27] and incubated for 24 to 48 h at 35°C.

Salmonella spp. were enumerated using a 3-dilution, 3-tube MPN [28] in which 1.0, 0.1, and 0.01 g of homogenized litter was suspended in tryptic soy broth [29] and incubated at 35°C overnight. An aliquot of 0.5 mL was transferred from each tube to Rappaport-Vassiliadis R10 broth [30] and incubated at 42°C for 24 to 48 h. Positive tubes were subsequently transferred (0.1 mL × 3 for each well) to 6-well cell culture plates [31] containing modified semisolid Rappaport-Vassiliadis agar [32]. Each well of the 6-well culture plates represented 1 inoculated tube in the MPN enumerated analysis. Presumed positive samples were streaked onto Hektoen Enteric agar [33] and incubated overnight at 35°C. Dark blue and black colonies were considered positive and confirmed using PCR.

Representative bacterial isolates from each sample location were preserved in 15% glycerol tryptic soy broth [29] and stored at −80°C for PCR confirmation. Each location was represented by a preserved bacterial isolate for a total

Table 1. Primers used for species-specific confirmation

Bacteria	Primers	Primer sequence 5′ to 3′	Control isolate	Reference
Salmonella enterica	inv-f	CTGTTGAACAACCCATTTGT	*S. enterica* Typhimurium ATCC 14028	[55]
	inv-r	CGGATCTCATTAATCAACAAT		
Staphylococcus spp.	Staph756F	AACTCTGTTATTAGGGAAGAACA	*Staphylococcus aureus* ATCC 25923	[56]
	Staph750R	CCACCTTCCTCCGGTTTGTCACC		
Listeria monocytogenes	prs-F-Lys	GCTGAAGAGATTGCGAAAGAAG	*L. monocytogenes* ATCC 51722	[57]
	prs-R-Lys	CAAAGAAACCTTGGATTTGCGG		
Enterococcus spp.	tuf-ent1F	TACTGACAAACCATTCATGATG	*Enterococcus faecalis* ATCC 19433	[58]
	tuf-ent2R	AACTTCGTCACCAACGCGAAC		
Campylobacter spp.	ceu-E – f	CCTGCTACGGTGAAAGTTTTGC	*Campylobacter jejuni* ATCC 33560	[55, 59]
	ceu-E – r	GATCTTTTTGTTTGTGCTGC		

of 192 isolates per bacteria if identified in litter. Twenty-five percent of all presumptive bacterial isolates were confirmed by PCR using species-specific primers (Table 1). Prior to mixing PCR reactions, 1 bacteria colony was inoculated into 200 µL of sterile deionized water and heated for 15 min at 95°C. The cell debris was centrifuged at 5,000 × *g* for 5 min at room temperature (22°C). The supernatant was used as a template for bacterial identification. Bacterial isolates were confirmed using a PCR mixture of 5 µL of 10× PCR buffer [34], 5 µL of 25 m*M* $MgCl_2$ [34], 1 µL of 10m*M* dNTPs mix [35], 0.3 µL of Amplitaq Gold (5U/µL) [34], 1 µL of each associated forward and reverse primer, 26.7 µL of PCR H_20 [36], and 10 µL of lysed bacteria isolate per reaction. Polymerase chain reaction was performed in a Thermo-Scientific Hybaid 0.2G thermocycler [37].

Antibiotic Resistance Profiles

This broiler operation was not actively administering antibiotics in a prophylactic manner. The only antibiotics administered to broilers, if any, were therapeutic; however, the antibiotics used and doses were not available. Representative isolates taken before flock placement (wk 0) and at final harvest (wk 6) of each flock were analyzed using the Kirby-Bauer technique for sensitivity to 12 antibiotics ranging from narrow to broad spectrum and encompassing 8 classes of antibiotics (Table 2) [38]. Isolates were plated to Mueller Hinton [39] (*Staphylococcus* spp.), tryptic soy agar (*Enterococcus* spp., *L. monocytogenes* and *Salmonella* spp.), or 5% sheep blood tryptic soy agar (*C. perfringens*) in 150-mm Petri dishes and were stamped with BBL Sensidisc antibiotics using a BBL antibiotic disc dispenser [40]. *Staphylococcus* spp., *Enterococcus* spp., and *L. monocytogenes* isolates were aerobically incubated for 16 to 24 h at 35°C. *Clostridium perfringens* plates were placed in an-aerobic Anoxomat chambers and incubated for 16 to 24 h at 44.5°C. Zones of inhibition (mm) were manually measured. *Staphylococcus aureus* ATCC 25923 [41], *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were included as antibiotic effectiveness quality controls. All bacteria were analyzed to determine resistance to ampicillin, amikacin,

Table 2. Antibiotic class resistance for bacterial-group isolates collected from broiler litter

Antibiotic class	*Staphylococcus* spp. n = 48	*Enterococcus* spp. n = 48	*Listeria monocytogenes* n = 22	*Clostridium perfringens* n = 48	*Salmonella enterica* n = 23
Penicillin[1,2]	0,0	0,1*	0,1*	2,3*	3*†
Cephalosporin[3]	0	15*	7*	2*	†
Glycopeptide[4]	0	8*	0	2*	†
Peptide[5]	†	†	†	†	2*
Macrolide[6]	12*	35*	9*	37*	†
Aminoglycoside[2,7]	0,0,0,6*	47,46,48,48*	2,0,0,0*	45,46,48,48*	1,10,0,1*
Tetracycline[8]	1	11*	5*	40*	10*
Quinolone[9]	1	13*	4*	3*	1*
Isolates with 2 class resistance	1	41	10	45	10
Isolates with 3 or more class resistance	0	16	4	32	0

[1]Penicillin (penicillin, ampicillin).

[2]Two or more values represents the number of resistant isolates for antibiotic classes in which more than one antibiotic was tested.

[3]Cephalosporin (cephalothin).

[4]Glycopeptide (vancomycin).

[5]Peptide (polymixin b).

[6]Macrolide (erythromycin).

[7]Aminoglycoside (amikacin, gentamicin, neomycin, kanamycin).

[8]Tetracycline (tetracycline).

[9]Quinolone (ciprofloxacin).

*Multiple class antibiotic resistance included for at least one isolate.

†Bacterial group is not susceptible to this class of antibiotic.

gentamicin, neomycin, kanamycin, tetracycline, and ciprofloxacin antibiotics. *Staphylococcus* spp., *Enterococcus* spp., *L. monocytogenes*, and *C. perfringens* were additionally tested for susceptibility to penicillin, cephalothin, vancomycin, and erythromycin. *Salmonella enterica* was also tested for resistance to polymixin B. Classification of resistance was determined using zone diameters published by the National Committee for Clinical Laboratory Standards [42, 43].

Mortality Data

As part of daily broiler house maintenance, the grower surveyed the house and removed dead birds. The grower kept a daily count of broiler mortalities removed from each house. Daily counts were totaled to determine the numbers of mortalities for each house and week sampled.

Statistical Analysis

The SAS Enterprise Guide 4.3 [44] program was used for all statistical analyses. All quantitative values (cfu or MPN/100 mL) were transformed by addition of 1 to convert zeros to positive numbers and $\log_{10}$ transformed. Chi-squared analysis was used for *L. monocytogenes* and *S. enterica* binomial data to determine effect on presence or absence ($\alpha = 0.05$). An ANOVA was performed for each bacteria of interest to compare the effects of sample location, broiler age, and flock. Analysis of *S. enterica* quantitative data (MPN/100 mL) was performed on positive samples only to achieve data normality as required by statistical analyses. Statistical differences between means were compared with Fisher's LSD at a probability level of 0.05. A total of 192 litter samples were collected and grouped according to variable or treatment factor investigated. One-way ANOVA were used to assess each effect of treatment factor independently. Effect of location was assessed by grouping all flock samples by location per flock per house half (north or south; n = 24), ignoring effect of flock. Effect of location per flock (n = 48), ignoring effect of house half, was also assessed. Broiler age was analyzed in the same manner as

location but replacing the independent variable with sampling week. Seasonal (flock) analysis was assessed for all data by grouping each variable according to flock.

RESULTS AND DISCUSSION

Salmonella enterica, *L. monocytogenes*, *Staphylococcus* spp., and *Enterococcus* spp. were detected during the analysis of spatial and temporal differences across 3 broiler flocks. *Salmonella enterica* and *L. monocytogenes* were isolated, but not at every location. Of the 192 samples analyzed, 28 *Salmonella*-positive and 47 *Listeria*-positive samples were identified from 3 flocks. *Staphylococcus* spp., *Enterococcus* spp., and *C. perfringens* were isolated from all locations. *Campylobacter* spp. was not detected from any sample collected. Prior to analyzing the effects of age, location, and other studied variables, a portion of all isolates (25%) collected from litter were confirmed via PCR. A total of 192 potential isolates were collected for each bacteria analyzed, therefore, one-fourth of all studied bacteria were randomly selected and confirmed. Of the 25% of isolates selected, all were confirmed using species-specific primers.

House design and broiler management practices impart natural spatial variability throughout the broiler house. This variability produces different microbial niches. Typically one end of the house, the nonbrood end, is equipped with massive exhaust fans that draw fresh air through the house. The other end of the house, the brood end, is used to brood the baby chicks at the beginning of each new flock grow-out period. The brood end typically has a large door that is closed during broiler placement, but opened for equipment entrance during harvesting of broilers due to the all-in/all-out method and subsequent management of litter between flocks. During brooding, the young chicks (0–2-wk-old) are confined to the brood end half of the house, which is partitioned off to reduce heating costs. As the birds increase in size the partitions are removed, the full house is available to the broilers. Thus, the nonbrood end houses the broilers from 2-wk-old until harvest (6–7-wk-old), whereas the brood end has broilers from d 1 through harvest. This 2-wk differential suggests inherent differences in the litter between the 2 halves of the house.

Some areas of the house are more subject to litter caking, the compaction of bedding material and excreta in areas where broilers congregate. Litter is typically decaked between flocks. This process removes the top caked layer that is higher in excreta and moisture. Differential caking and decaking produce distinct niches that favor distinct microbial populations. The area immediately adjacent to the wall of the house is unique, because equipment constraints preclude complete litter removal during decaking; often leaving 30- to 60-cm wide strips of accumulated cake along the walls. Litter in high traffic areas near WL and around FL have more caking, but cake near WL has higher moisture content.

Effect of Location

Sample location had an effect on some bacterial levels and presence. Though there seemed to be unique management characteristics that could affect microbial constituents as described in the above paragraphs, no differences were noted for any of the surveyed bacteria when comparing brood versus nonbrood halves (data not shown). When analyzing moisture content, the mean moisture content of EW (59%) were statistically highest among locations, and the increased moisture may have favored specific bacterial populations in these areas (Table 3). Among surveyed bacteria, *S. enterica* and *Staphylococcus* spp. levels were significantly associated with site location with a statistical *P*-value of 0.0292 and 0.0405, respectively (Figures 1 and 2). Specifically, EW samples were found to harbor statistically higher levels of *S. enterica*, whereas *Staphylococcus* spp. levels were lower near FL. Approximately 24% of samples collected throughout all 3 flocks were positive for *L. monocytogenes* with no association directly to location in the house. Though commonly associated with poultry, *Campylobacter* levels were below detection limits in all samples throughout the study. Environmental *Campylobacter* can be present in a reduced metabolic, viable but nonculturable state, which can prevent its isolation in harsh environments yet still provide detection at processing plants where conditions improve [45–47]. *Enterococcus* spp. were found throughout the house at levels of 8 to 10 $\log_{10}$ cfu/kg of litter with no effect of location (Figure 3). Simi-

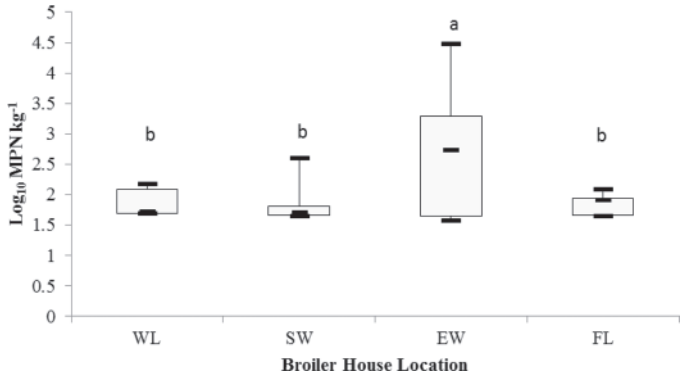

Figure 1. *Salmonella enterica* levels associated with mean positive values of each location within the broiler house. Broiler house locations represented are water lines (WL), side walls (SW), end walls (EW), and feeder lines (FL). Different letters (a,b) represent mean statistical differences in *S. enterica* MPN/kg of litter for all locations ($P \leq 0.05$). Error bars represent SD; n = 48/sample location.

larly, levels of *C. perfringens* were not affected by location and averaged 7.5 $\log_{10}$ cfu/kg of litter (Figure 3).

Effect of Broiler Age

Broiler age had a significant effect on nearly all studied microbes ($P < 0.05$). *Salmonella enterica*, *L. monocytogenes*, *Enterococcus* spp., *Staphylococcus* spp., and *C. perfringens* levels were all associated with broiler age. Litter moisture content, litter temperature, and broiler mortalities were also associated with broiler age (Table 4). *Salmonella enterica* was more commonly isolated before flock placement (36%). Increased isolation of *S. enterica* before flock placement may be attributed to lower competitive exclusion. In the current study, other assayed bacterial populations increased each week coincidental with decreases in *Salmonella* spp. levels, suggesting a possible loss in environmental niche. Likewise, as the broilers age, their immunity improves, which may reduce gut and fecal pathogen colonization, thus also reducing their levels in litter.

Listeria monocytogenes presence was also associated with broiler age, when broiler age by flock was considered. Chi-squared analysis per flock indicated *L. monocytogenes* presence was higher for flock 1 and 2 during early (wk 0) and midweeks (wk 2 and 4), but only wk 6 for flock 3. This shift may be due to seasonal influences. The moisture content was found to be lower during the winter flock. *Listeria monocytogenes* may be more sensitive to this drop in litter moisture.

Throughout the study, *Staphylococcus* spp. were present at 5 to 9 $\log_{10}$ cfu/kg of litter greater than any other bacteria investigated in the study (Figure 3). A gradual per week increase in staphylococci was seen for all locations, with statistically significant increases occurring between wk 2 and 4 (Figure 4). *Enterococcus* spp. levels were affected by broiler age as well; the difference was statistically significant between all sampling weeks except for wk 2 and 4 (Figure 3).

For *C. perfringens* levels, wk 0 through 4 remained relatively constant; wk 6, for all flocks, was significantly higher than other sampling times (Figure 3). *Clostridium perfringens* increased approximately 1 $\log_{10}$ cfu/kg of litter from wk 4 to 6. No significant increases were noted for flock 1 or 2 when comparing intraflock litter moisture content changes for each sampling week; however, flock 3 had significantly

Table 3. Mean moisture content of broiler litter for each location[1]

Site	Moisture content (%)
Water line	42.6[ab]
Side wall	36.9[b]
End wall	59.0[a]
Feeder line	26.8[b]

[a,b]Means within a column with different superscripts differ significantly at $P < 0.0001$.

[1]n = 48/location.

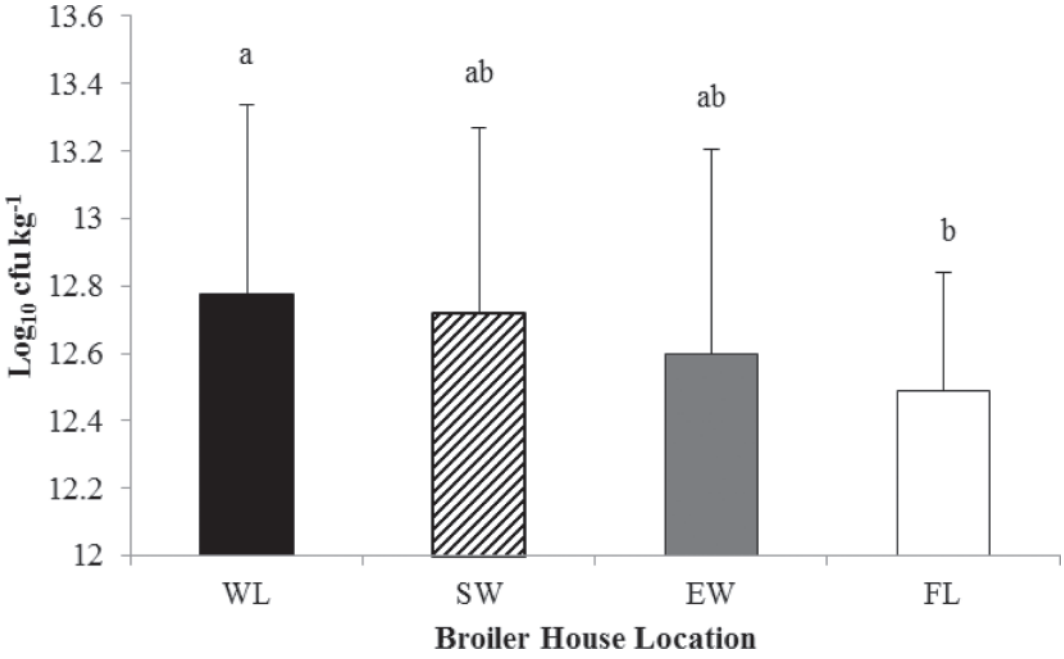

Figure 2. S*taphylococcus* spp. levels associated with each location within the broiler house. Broiler house locations are water lines (WL), side walls (SW), end walls (EW), and feeder lines (FL). Different superscripts (a,b) represent statistical differences in *Staphylococcus* spp. cfu/kg of litter for all locations ($P \leq 0.05$). Error bars represent SD; n = 48/sample location.

drier litter due to the use of heaters to maintain temperatures within the house in winter months. The litter moisture content was not statistically higher for each week for all flocks, but an upward trend may have allowed for favorable anaerobic conditions to permit *C. perfringens* to proliferate.

Mortality rates peaked at wk 2 and 6 across all flocks. Mortality numbers from wk 2 are associated with initial broiler placement and may have little to do with litter or house environment. Young broilers are more susceptible to disease due to a less developed immune system and lack the necessary sustaining microflora in the gut that competitively excludes pathogens from overwhelming the gastrointestinal tract [48]. *Clostridium perfringens* causes necrotic enteritis and necrotizing fasciitis in poultry and is a major contributor to broiler mortality, which may explain the increased mortality rates for wk 6, though no broiler was examined to confirm [49].

Seasonality (Flock)

Each flock was presented with different seasonal (environmental) characteristics. Recog-

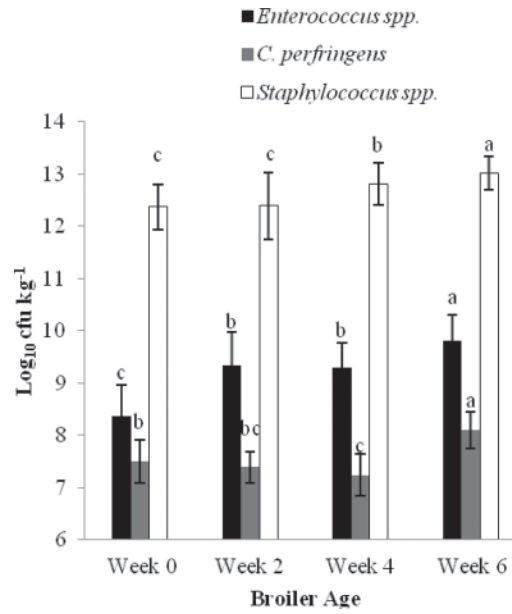

Figure 3. *Enterococcus* spp., *Clostridium perfringens*, and *Staphylococcus* spp. levels associated with broiler age. Different superscripts (a–c) represent statistical differences in *Enterococcus* spp., *C. perfringens*, and *Staphylococcus* spp. cfu/kg of litter for broiler age ($P \leq 0.0001$). Each bacterial group was analyzed independently. Error bars represent SD; n = 48/week.

Table 4. Mean litter characteristics associated with broiler age and broiler mortality per week

Broiler age[1]	Litter moisture content[2] (%)	Litter temperature[2] (°C)	Broiler mortality[3] (no.)
0 wk	49.2[a]	26.4[c]	54[b]
2 wk	31.6[b]	29.2[b]	272[a]
4 wk	37.3[ab]	31.0[ab]	72[b]
6 wk	47.2[a]	31.9[a]	308[a]

[a–c]Means within a column with different superscripts differ significantly at $P < 0.05$.

[1]Broiler litter samples were combined from all flocks and analyzed according to broiler age.

[2]n = 48 broilers.

[3]Mean number of deceased broilers removed each sampling week.

nizing that each season is only represented by 1 flock, the analysis to determine changes across flocks are described as seasonal differences to offer explanation of these differences. A part of broiler house maintenance is regulating house ambient temperature to reduce seasonal effects on the birds. The heating and cooling systems maintain approximate constant temperatures within the house; however, climatic factors outside the house may affect house environmental conditions. The moisture and temperature of the litter can be altered due to outside ambient conditions. This was the case with litter temperature, which was significantly lower for flock 3 (winter) for all areas of the house (data not shown). The mean moisture content of the litter during the winter flock was lower than the other 2 flocks (Table 5). The drier litter may be due to the heaters used during this time of grow-out. Opara et al. [50], when investigating the presence of pathogens in poultry litter, found a direct correlation to increased water activity and the

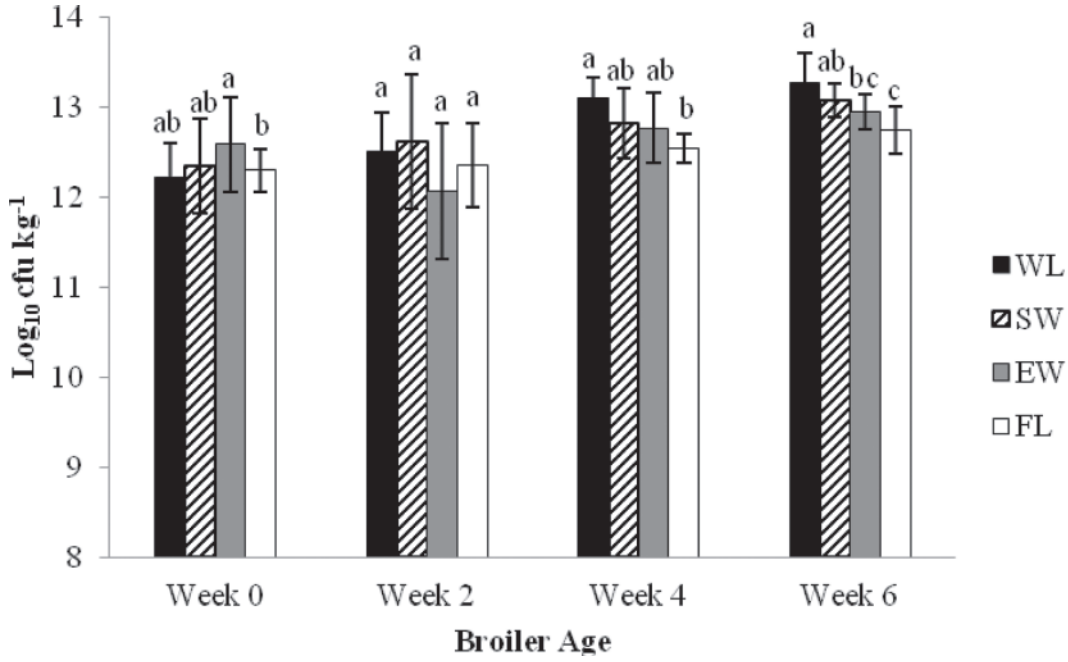

Figure 4. *Staphylococcus* spp. levels associated with broiler age per location. Broiler house locations are water lines (WL), side walls (SW), end walls (EW), and feeder lines (FL). Different superscripts (a–c) represent statistical differences in *Staphylococcus* spp. cfu/kg of litter for broiler age ($P \leq 0.05$). Statistical analysis was associated with mean staphylococci levels within each week from all locations. Error bars represent SD; n = 48/week.

Table 5. Seasonal[1] differences of mean litter moisture content, mean levels of *Staphylococcus* spp., and percentage of positive samples of *Salmonella enterica* and *Listeria monocytogenes* (n = 64)

Flock[1]	Moisture (%)	*Staphylococcus* spp. (log_{10} cfu/kg)	*S. enterica* positives (%)	*L. monocytogenes* positives (%)
1	40.0[ab]	12.4[b]	15*	27*
2	50.6[a]	12.8[a]	11*	13*
3	33.4[b]	12.6[a]	2*	7*

[a,b]Mean statistical differences within a column with different superscripts differ significantly at $P < 0.05$.

[1]Flock 1, 2, and 3 represent summer, fall, and winter, respectively.

*$P \leq 0.05$.

ability to isolate litter microbes. The drier litter during flock 3 in the present study could explain why fewer bacteria were isolated.

Chi-squared analysis among flocks indicated an association between seasonality and *S. enterica* isolation ($P = 0.0038$). When comparing the percentage of *S. enterica* isolates recovered, 54% (15/28) of all positive samples were collected during flock 1 (summer) followed by 39 (11/28) and 7% (2/28) from flock 2 (fall) and 3 (winter), respectively. These findings were consistent with research which found that *Salmonella* is more likely to persist throughout the flock if the pathogen is detected before flock placement [51–53]. For flocks 1 and 2, *S. enterica* was detected more frequently at wk 0. A significant difference was associated with *L. monocytogenes* isolation and seasonality (Table 5). The distribution of all *Listeria*-positive isolates across flocks 1, 2, and 3 was 57, 28, and 15%, respectively, with a total of 47 total positive samples. *Staphylococcus* spp. levels were highest during flock 2, whereas *C. perfringens* and *Enterococcus* spp. were not affected by seasonal changes (Table 5).

Animal welfare is a major concern in the broiler production industry. Increasing feed conversion to broiler weight and decreasing mortality per flock are the ultimate goals for the broiler growers. In the present study, mortality varied seasonally as each successive flock had a higher mortality rate. Total mortality rates were 2.3, 3.5, and 8.5%, for flocks 1, 2, and 3 respectively. The causes of increasing mortality in successive flocks were not identified in the present study, and it is possible that the microbes or conditions responsible were not investigated. Future research involving molecular analysis of spatial microbial communities may give more information on broiler health and mortality.

Antibiograms

No apparent shifts in *C. perfringens* antibiograms can be seen when comparing antibiotic resistance profiles from isolates taken before flock placement until the final sampling week of flock grow-out. *Enterococcus* antibiograms had a greater number of resistant isolates for flocks 1 and 2 than flock 3 for cephalosporin (cephalothin), glycopeptide (vancomycin), tetracycline (tetracycline), and quinolone (ciprofloxacin). *Enterococcus* was the only pathogen for which location may have influenced resistance. Twenty-five percent of the *Enterococcus* spp. isolates taken from the ends of the broiler house were resistant to cephalosporin and 16% were resistant to vancomycin. One quarter of *Staphylococcus* spp. isolates were intermediately or completely resistant to erythromycin (Table 2). Most *Staphylococcus* spp. isolates were resistant to only one class of antibiotics, but one (EW-south, wk 0, flock 3) exhibited multiclass resistance to macrolide and aminoglycoside classes. No difference in antibiotic resistance was observed for broiler age or seasonality. Most *Staphylococcus* spp. isolates (29/48) were predominantly susceptible to all tested antibiotics. *Salmonella enterica*, *Enterococcus* spp., *C. perfringens*, and *L. monocytogenes* isolates possessed multiple antibiotic resistance (**MAR**) profiles (Table 2). Kelley et al. [54] and Brooks et al. [11] determined similar results of MAR in poultry house isolates. Brooks et al. [11] concluded that these MAR profiles were contained within the house, as isolates from outside the poultry house did not share the same MAR properties. Future research should give more attention to antibiotic resistance profiles and the selective pressures which influence MAR bacterial persistence in the poultry house environment.

CONCLUSIONS AND APPLICATIONS

1. All bacterial pathogens, *S. enterica*, *L. monocytogenes*, *Enterococcus* spp., *C. perfringens*, and *Staphylococcus* spp. were isolated in all areas of the house. *Staphylococcus* spp. were found at significantly lower levels at FL, and *Campylobacter* spp. was not isolated at any location. After examining spatial differences, few associations could be determined based solely on location, though *Salmonella enterica* was found to be more commonly associated with the EW of the house. Isolating *S. enterica* in 15% of the 192 samples demonstrates that this pathogen continues to be problematic and may deserve site-specific litter treatment to reduce its levels while keeping costs down for the farmer.
2. When investigating antibiotic-resistant profiles, most staphylococci were inhibited by all tested antibiotics. *Salmonella enterica*, *C. perfringens*, *L. monocytogenes*, and *Enterococcus* spp. isolates were resistant to not only multiple antibiotics but multiple classes as well. The MAR profiles, for the studied pathogens, provide evidence that antibiotic resistance may continue, despite limited farm antibiotic use.
3. Broiler age was the most significant factor affecting bacterial proliferation; temporal differences appear to be the more relevant focus for effective pathogen reduction.
4. Future studies investigating the temporal-spatial effect on litter microbial communities may provide useful data and insight into cost-effective pathogen control.

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